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FURTHER OBSERVATIONS ON THE EOSIN-METHYLENE BLUE AGAR¹

By MAX LEVINE²

At the Council Bluffs meeting of this section, in 1917, the author had the honor and pleasure to make public a simplified eosin-methylene blue agar to be employed in the confirmation of the presumptive test for *B. coli* in water analysis. The medium consists of 1 per cent peptone, 0.2 per cent dipotassium phosphate, 1.5 per cent agar, and 1 per cent lactose with the dyes eosin and methylene blue as indicators. The advantages claimed for the medium were, first, extreme simplicity of preparation, as neither adjustment of reaction nor filtration is required, and second, that *B. coli* and *B. aerogenes*, two organisms which have been confused in the past and which may be of different sanitary significance, are easily differentiated.

It was stated at the time that 96.9 per cent of *B. coli* and 82.4 per cent of *B. aerogenes* were correctly diagnosed from their appearance on eosin-methylene blue agar. The reliability of this differentiation has since been checked by the author, in 1918 and 1919, at the Central Medical Department Laboratory of the American Expeditionary Forces in Dijon, France, where he found 94.2 per cent of the colonies picked as *B. coli* and 85.5 per cent of supposedly *B. aerogenes*, confirmed by further tests.

The medium has been employed with success and satisfaction in the State Water Laboratories at Iowa City; in Army Laboratory Schools for officers at Ft. Leavenworth and Yale University; at the Army Medical School, Washington, D. C.; and a number of water laboratories overseas.

In the original report, only one type of *B. coli* colony was described. Subsequent extensive observations have disclosed three distinct types of colony growth of this organism. The first, which is regarded

¹ Read before the Iowa Section, November 5, 1920. Discussions are desired and should be sent to the Editor.

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as the typical *B. coli* and which constitutes 95 to 98 per cent of the types encountered, has the following characteristics:

Isolated colonies are 2 to 3 mm. in diameter and they show very little tendency to run together. The colonies are slightly raised, the surface is flat or slightly concave, and the edge is even and regular when viewed with the naked eye. By transmitted light, they appear dark, and have almost black centers which extend more than three-fourths of the distance across the diameter of the colony. By reflected light they appear dark, button-like, often concentrically ringed and show a distinct, characteristic, greenish, metallic sheen.

Types of colonies of B. coli on eosin-methylene blue agar

SIZE	I. WELL ISOLATED COLONIES 2-3 MM. IN DIAMETER	II. SAME AS I	III. LARGER THAN I USUALLY 3-4 MM. DIAMETER
Confluence	Neighboring colonies do not run together	Same as I.	Same as I
Elevation	Slightly raised	Slightly raised	Flat,—effuse. Appears almost as if part of the surface of medium
Surface	Flat or slightly concave	Usually slightly convex	Flat
Edge (viewed with naked eye)	Regular, entire	Same as I	Irregular, crenated, or lacerated
Appearance by transmitted light	Dark, almost black centers which extend three-fourths across the diameter of colony, surrounded by a narrow lighter zone	Purple centers	Like I but outer zone is irregular and often much broader
Appearance by reflected light	Dark button-like, often concentrically ringed, with a greenish metallic sheen	Wine colored, metallic sheen not present	Distinct greenish metallic sheen like I

A second type of *B. coli* colony encountered resembles the typical form just described with respect to size and elevation, but there is no metallic sheen, the colonies being wine-colored.

The third variety of colon colony appearing on this medium is somewhat larger (3 to 4 mm.) than the typical form described. It grows effusely, or flat, the edge is crenated or irregular, and the central portion has a distinct metallic sheen.

Several varieties of *B. aerogenes* have also been observed. The most frequently encountered colony type and the one which was originally described is as follows:

Well isolated colonies are larger than *B. coli*, usually 3 to 5 mm. in diameter, and neighboring colonies will quickly run together forming a limy mucoid-appearing mass. The colonies are moderately raised, markedly convex, while the centers are a light to a dark brown and relatively small, less than one-half the diameter of the colony. By reflected light, they appear much lighter than *B. coli* and there is no metallic sheen.

The second type of *B. aerogenes* encountered produces colonies somewhat larger than the one described above. The center is very dark, wine-colored, and relatively large (often being more than three-fourths of the diameter of the colony) and there is a glossy metallic sheen in young cultures. Confluence is very marked.

A third type of *B. aerogenes* observed is a small colony, often smaller than *B. coli*. The colonies are raised but the center drops precipitately like a crater. By reflected light, the small dark central portion may show a metallic sheen.

Whether these different types of colonies are of different sanitary significance has not as yet been determined but it is apparent that the medium affords a means of differentiating varieties of *B. aerogenes* and *B. coli* which the author believes has not been attained with any of the other media employed for confirming the presumptive test. It has also been observed that *B. cloacae* and particularly *Ps. pyocyaneus*, which have occasionally been mistaken for *B. coli* on the standard Endo medium, grow very poorly on eosin-methylene blue agar, forming colonies which are not likely to be mistaken for either *B. coli* or *B. aerogenes*.

Test tubes may be substituted for petri dishes if desired. The value of such a change is, first, reduction of expense, as only 3 or 4 cc. of medium is needed for each test tube, while about 15 cc. is usually employed with a petri dish; and, second, test tubes may be

Types of colonies of B. aerogenes on eosin-methylene blue agar

SIZE	I. WELL ISOLATED COLONIES 3-5 MM. IN DIAMETER	II. SLIGHTLY LARGER THAN I. 3-6 MM. IN DIAMETER	III. SMALLER THAN I. 1½-3 MM. DIAMETER
Confluence	Neighboring colonies run together or fuse	Like I	Colonies remain isolated and distinct, little tendency to fuse
Elevation	Moderate raised	Considerably raised	Slightly raised
Surface	Convex	Convex	Center drops precipitately like crater
Edge (naked eye)	Regular	Regular	Regular
Appearance by transmitted light	Light to dark brown centers which are relatively small (less than one-half diameter of the colony) surrounded by a broad grey or pink zone	Very dark, large wine colored centers often three-fourths diameter of colony	Small pinhead or slightly larger center, light to a dark brown
Appearance by reflected light	Metallic sheen not present. Colonies appear glossy and mucoid	Like I but very strong green metallic sheen, particularly in young cultures	Colonies distinct, comparatively dull and the crateriform center may have a metallic luster

stored for long periods, whereas the medium in petri dishes would have to be prepared at intervals of a week or less. The method of preparing test tubes is briefly as follows:

To 100 cc. of sterile melted phosphate agar is added 5 cc. of a 20 per cent lactose, 2 cc. of 0.5 per cent aqueous methylene blue solution and 2 cc. of a 2 per cent aqueous eosin. The medium is then poured aseptically into sterile test tubes and allowed to solidify so as to give a long slant without a butt. To confirm the presumptive

test, inoculation is made on the surface of these slants with a straight platinum needle. If desired the medium may be placed in test tubes and sterilized in the Arnold or in the autoclave in the ordinary manner. In that case it will be found that the medium, when it comes out of the sterilizer, is fluorescent like eosin; but as it cools, the typical wine color returns. The author prefers to prepare tubes as described above.

The author has felt for sometime that before much light will be thrown on the true relative incidence of *B. coli* and *B. aerogenes* in water, sewage, etc., it will be necessary to first modify our preliminary enrichment media or other conditions so as to enable an investigator to isolate or suppress *B. coli* or *B. aerogenes* at will.

Vigor of growth of B. coli and B. aerogenes on eosin-methylene blue agar with various concentrations of brilliant green. (Observations after twenty-four hours)

CULTURE	CONCENTRATION OF BRILLIANT GREEN				
	None (control)	1:400,000	1:300,000	1:200,000	1:100,000
<i>B. aerogenes</i> 8b.....	+++	+++	+++	+++	++
<i>B. aerogenes</i> 11.....	+++	+++	++	+	+
<i>B. aerogenes</i> 13.....	+++	+++	+++	+	+
<i>B. aerogenes</i> 17d.....	+++	+++	++	++	+
<i>B. coli</i> 19b.....	+++	—	—	—	—
<i>B. coli</i> 20b.....	+++	—	—	—	—
<i>B. coli</i> 21c.....	+++	+	+	—	—
		(Faint atypical)	(Faint atypical)		
<i>B. coli</i> 22.....	+++	+	+	—	—
		(Faint atypical)	(Faint atypical)		

Attempts to devise media which will inhibit *B. aerogenes* and at the same time permit *B. coli* to grow have not been very successful. By the addition of 1:300,000 to 1:400,000 brilliant green to eosin-methylene blue agar, the growth of *B. coli* may be markedly suppressed; whereas *B. aerogenes* grows very luxuriantly.

The researches of Rogers and his associates in the Bureau of Animal Industry, United States Department of Agriculture, and those of the Iowa Engineering Experiment Station, which have been adequately confirmed by many investigators, have shown that *B.*

coli is characteristic of the intestinal tract of man and the higher animals; whereas *B. aerogenes*, which has been formerly confused with *B. coli*, is very rarely encountered in human or animal excreta but constitutes the predominating type in soil and on grains. These two organisms, it is believed, should not be considered of the same sanitary significance, and water works men are naturally interested in obtaining information as to the relative incidences of these two bacteria in raw and treated water. The eosin-methylene blue agar is suggested as a simple and reliable medium for the detection of *B. coli* and at the same time differentiating it from its less objectionable relative, the *B. aerogenes*.